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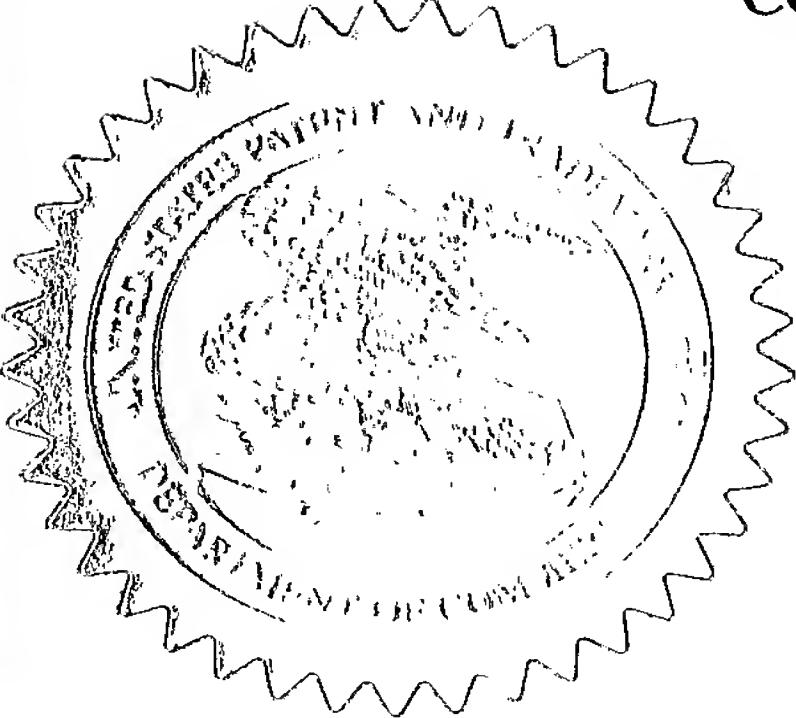
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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

**Express Mail Label No.****INVENTOR(S)**

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Toshihiro	Akaike	Nishitokyo-shi, Tokyo, Japan

Additional inventors are being named on the **2nd** separately numbered sheets attached hereto**TITLE OF THE INVENTION (500 characters max)****Mg<sup>2+</sup>-inspired generation of nano-apatite for high efficiency transfection of mammalian cells**Direct all correspondence to: **CORRESPONDENCE ADDRESS** Customer Number:**OR**

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**ENCLOSED APPLICATION PARTS (check all that apply)**

<input checked="" type="checkbox"/> Specification Number of Pages	9	<input type="checkbox"/>	CD(s), Number
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[Page 1 of 2]

Respectfully submitted,

SIGNATURE Toshihiro AkaikeTYPED or PRINTED NAME Toshihiro AkaikeTELEPHONE +81-424-22-2774Date December 26, 2003REGISTRATION NO. \_\_\_\_\_  
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Docket Number **TA - 003**

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# **Mg<sup>2+</sup>-inspired generation of nano-apatite for high efficiency transfection of mammalian cells**

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## Abstract

Transfer of desirable genetic sequences into mammalian cells is an essential tool for analysis of gene structure, functions and regulation, and industry-based production of therapeutically important proteins and pivotal for gene therapy and DNA vaccination strategies. Considering some severe limitations of viral systems including immunogenicity, carcinogenicity and so on, synthetic non-viral systems are highly desirable in the above applications. However, existing non-viral techniques are extremely inefficient compared to the viral ones. So we report here on the development of a highly efficient synthetic device for gene delivery and expression into mammalian cells, based on controlled growth of nano-apatite particles. Mg<sup>2+</sup> incorporation into the apatite particles caused significant inhibition of particle-growth, resulting in retention of nano-sized particles which contributed remarkably to the cellular uptake of DNA and its subsequent expression (>10-fold) compared with classical calcium phosphate co-precipitation, one of the most widely used transfection methods.

## Text

Significant efforts are now being made for the development of non-viral gene-delivery techniques as alternatives to the viral vectors for basic research and clinical medicine (1). Despite a wide variety of non-viral techniques existing with potential application in gene therapy, limited understanding of the molecular and cellular basis in gene transfer hinders the development of efficient technology. Co-precipitation of DNA with calcium phosphate which is based on hydroxyapatite, is one of the most commonly used non-viral vectors (2-13), having potential applications in gene therapy (2-3). Although inefficiency in particle-mediated uptake of DNA by the cells has been considered as a major barrier of low transgene expression in vitro and in vivo<sup>2,3,6,11,14-17</sup>, an effective way of manipulating particle growth kinetics at the molecular level has not been disclosed so far, which could overcome the hurdle dramatically. Here, we describe, for the first time, the generation of Ca-Mg phosphate precipitates which like Ca phosphate precipitates, adsorbed DNA, but unlike the latter, could prevent the growth of the particles to a significant extent, resulting in huge cellular uptake of DNA, followed by notably high transgene expression.

## Results and discussion

### Generation and chemical characterization of Ca-Mg phosphate particles

Addition of 0 to 140 mM Mg<sup>2+</sup> along with 125 mM Ca<sup>2+</sup> to Hepes-buffered solution (pH 7.05) containing 0.75 mM inorganic phosphate, followed by incubation at room temperature, resulted in microscopically visible particles (not shown here). As shown in

supplementary fig. 1, IR spectrum of Ca phosphate particles (generated in absence of  $Mg^{2+}$ ) suggests formation of hydroxyapatite as the peaks between  $1000 - 1100\text{ cm}^{-1}$  and  $550 - 650\text{ cm}^{-1}$  represents phosphate in the structure. X-ray diffraction patterns also shows the characteristic peak of hydroxyapatite (see supplementary fig. 2). To know the chemical composition of all types of the particles, elemental analysis was performed (Table 1 and 2) for sample 1, 2, 3, 4, 5, 6, 7 and 8, representing, respectively, 0, 20, 40, 60, 80, 100, 120 and 140 mM  $Mg^{2+}$  added for particle generation (described above). As shown in Table 1, with increase in  $Mg^{2+}$  concentrations in solution, particle-associated  $Mg^{2+}$  level increased upto ~3% with concomitant decrease in  $Ca^{2+}$  level whereas phosphorus (P) level remains almost fixed for sample 1 to 3 (~12%) and sample 4 to 8 (~16%), indicating precipitation of 2 different types of apatite. The molar ratio values (Table 2) indicate formation of hydroxyapatite with the formula  $Ca_{10-x}Mg_x(PO_4)_6(OH)_2$  for sample 1 to 3 and octacalcium phosphate (OCP) with the formula  $Ca_{4-x}Mg_x(PO_4)_3$  for sample 4 to 8, thereby suggesting that a high  $Mg^{2+}$  level drives the reaction to the formation of OCP (18-19).

### **Regulating growth kinetics and sizes of particles**

Turbidity determination of a particle suspension could be interpreted to analyse time-dependent particle growth, following nucleation in a supersaturated solution (11). As shown in Fig. 1-A, at 1min following mixing all of the components in Hepes-buffered solution (described above), turbidity declined continuously with increasing  $Mg^{2+}$  concentrations in the solution, suggesting clearly that incorporated  $Mg^{2+}$  slows down the growth of the particles to a significant extent. With incubation for additional periods (5 to 30 min), turbidity plot showed an up and down profile which could be explained with the notion that an increasingly high concentrations of  $Mg^{2+}$  (20 to 60 mM) could further induce the precipitation reaction depending on the incubation time, thus causing

an increment in turbidity for an increase in particle numbers and that with a more significant amount of Mg<sup>2+</sup> (80 to 140 mM), inhibition of particle growth played the major role for the sharp decrease in turbidity. In order to make a better understanding of how Mg<sup>2+</sup> inclusion into the particles contributes immensely to the reduction of the growth and consequently the sizes of the particles, we estimated the mean diameters of all types of particles during their growing stages. As shown in Fig. 1-B, at a period of time from 1 to 30 min following initiation of precipitation reaction, an increasing dose of Mg<sup>2+</sup> dramatically reduced the particle diameters from micro to nano level. Moreover, the figure enables us to predict a clear and reliable growth kinetics indicating that an increasingly high Mg<sup>2+</sup> incorporation could transform a fast growing particles to more slowly growing ones having size distribution in the nano-meter range. The strong inhibitory effect of Mg<sup>2+</sup> on particle growth could be explained by creation of a distorted atomic structure in hydroxyapatite upon replacement of Ca<sup>2+</sup> with Mg<sup>2+</sup>, that subsequently slows the growth of the particles (20).

### **High rate cellular uptake of DNA carried by nano-apatite**

Particle size is a crucial factor for successful gene transfer into mammalian cells; fine particles mediate an efficient gene transfer, whereas coarse ones do not (11, 13). Rapid growth of the particles resulting in sharp increase in diameter (Fig. 1-B) is thus a big hurdle which must be eliminated for efficient gene delivery and expression into the cells. Since Ca-Mg phosphate could block the growth and limit the sizes of the particles at a desirable level, we investigated DNA uptake in the cells, mediated by the particles. As shown in Fig. 2, internalization of DNA by Mg<sup>2+</sup>-free particles was inefficient and gradually decreased to the lowest level due to the growth of the particles (Fig. 1). On the contrary, strong fluorescence of PI (propidium iodide)-labeled DNA was observed inside the cells for Mg<sup>2+</sup>-containing particles (Fig. 2) which were sufficiently resistant

to growth (Fig. 1), indicating that DNA/Ca-Mg phosphate particles are efficiently endocytosed owing to their potential ability of blocking particle growth. The decline in uptake efficiency level for the particles generated with a high Mg<sup>2+</sup> dose (Fig. 2) indicates formation of insufficient amount of nano-particles (Fig. 1) since Mg<sup>2+</sup> beyond a level, could abolish precipitation reaction (18, 19).

#### **Notable level of transgene expression mediated by nano-apatite**

To reach the final goal of our strategy, we checked expression profile of a luciferase gene based on DNA/Ca-Mg phosphate particles isolated according to a specified timetable. Surprisingly, depending on the level of Mg<sup>2+</sup> and particle generation time, at least 10 to 40-fold higher luciferase expression could be detected compared with Mg<sup>2+</sup>-free particles. Such a high transfection efficiency could be solely attributed to the intrinsic property of Ca-Mg phosphate to significantly block the growing process and consequential generation of nano-sized particles (Fig. 1) needed for efficient cellular uptake of DNA. The profound effect of particle sizes on DNA delivery and subsequent expression could be clearly seen when the particles are allowed to grow for 30 min; Mg<sup>2+</sup> inclusion caused a remarkable transition of particle diameter from 2.5  $\mu$ m to 500 nm and finally enhanced gene expression efficiency by at least 40-times. Thus, instead of providing tremendous efforts for limited transfection activity by collecting the precipitates just after initiation of precipitation (11), Mg<sup>2+</sup>-regulated particle growth profiling could confer a highly flexible way of nano-apatite preparation/ and enable to establish a super-efficient gene delivery system for mammalian cells.

Considering the high impact of a traditionally and widely used transfecting agent like Ca phosphate precipitate in basic research laboratories, biotech companies for

production of recombinant cell lines and recently in gene therapy (2-3), our newly developed technology based on Ca-Mg phosphate nano-precipitate, would emerge as a tool of utmost importance in the above applications replacing the old one.

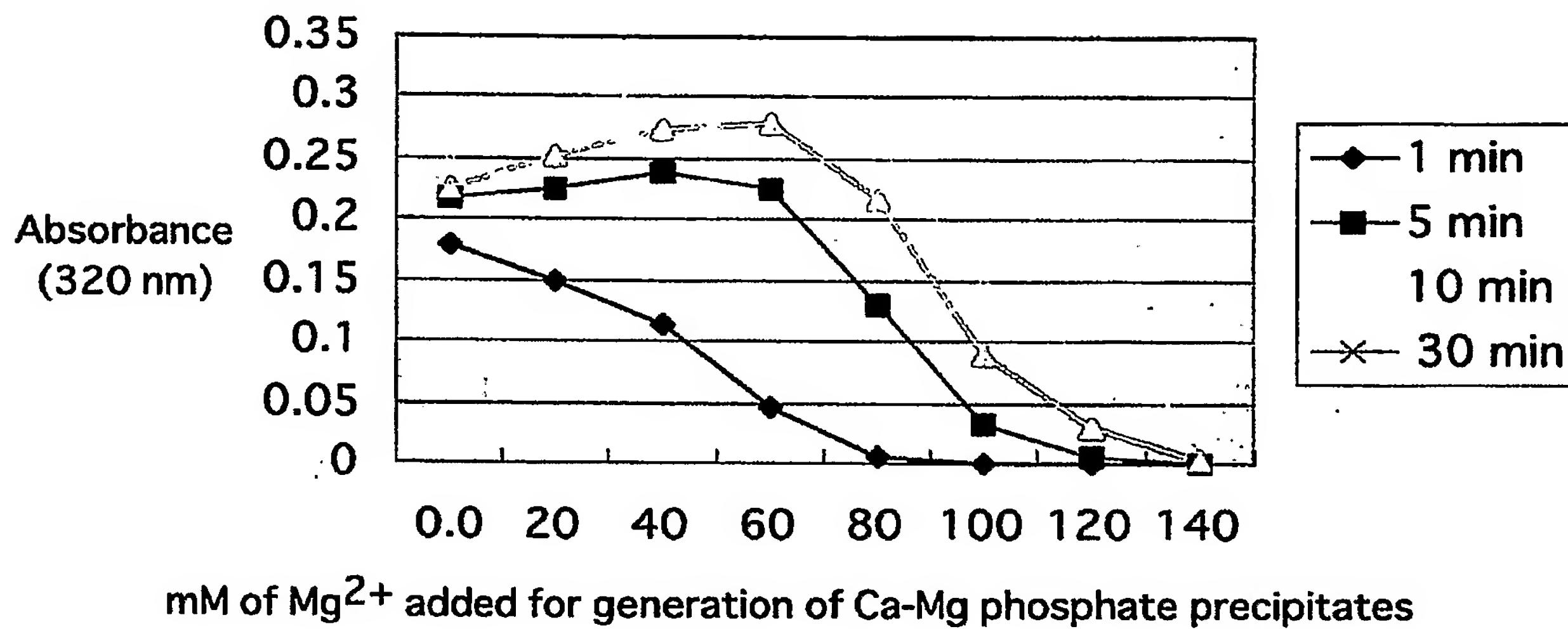
## References

1. Luo, D. & Saltzman, W. M. (2000) *Nature Biotechnology* 18, 33-37.
2. Fasbender, A., Lee, J.H., Walters, R.W., Moninger, T.O., Zabner, J. & Welsh, M.J. (1998) *J. Clin. Invest* 102, 184-193.
3. Toyoda, K., Andresen, J., Zabner J., Faraci, F. & Heistad, D. (2000) *Gene Therapy* 7, 1284-1291.
4. Graham, F. L. & A. J. van der Eb. (1973) *Virology* 52, 456-467.
5. Gorman, C., Padmanabhan, R. & Howard BH. (1983) *Science* 222, 551-553.
6. Chen, C. & Okayama, H. (1987) *Molecular and cellular biology* 7, 2745-2752.
7. Brash, D. E. et al. (1987) *Molecular & cellular biology* 7, 2031-2034.
8. Kjer, K.M. & Fallon, A.M. (1991) *Arch. Insect Biochem. Physiol.* 16, 189-200.
9. O'mahoney J. V. & Adams T. E. (1994) *DNA & cell biology* 13, 1227-1232.
10. Song, W. & Lahiri D. K. (1995) *Nucleic Acids Research* 23, 3609-3611.
11. Jordán, M., Schallhorn, A. & Wurm FM. (1996) *Nucleic acids research* 24, 596-601.
12. Lee, J. H. & Welsh, M. J. (1999) *Gene therapy* 6, 676-682.
13. Urabe, M., Kume, A., Kiyotake Tobita, K. & Ozawa, K. (2000) *Analytical Biochemistry* 278, 91-92.
14. Loyter, A., Scangos, GA. & Ruddle, FH. (1982) *Proc. Natl. Acad. Sci. USA* 79, 422-426.
15. Loyter, A., Scangos, G., Juricek, D., Keene, D. & Ruddle , F. H. (1982) *Experimental Cell Research* 139, 223-234.
16. Orrantia, E. & Chang, P. L. (1990) *Experimental cell research* 190, 170—174.
17. Batard, P., Jordan, M. & Wurm. F. (2001) *Gene* 270, 61-68.

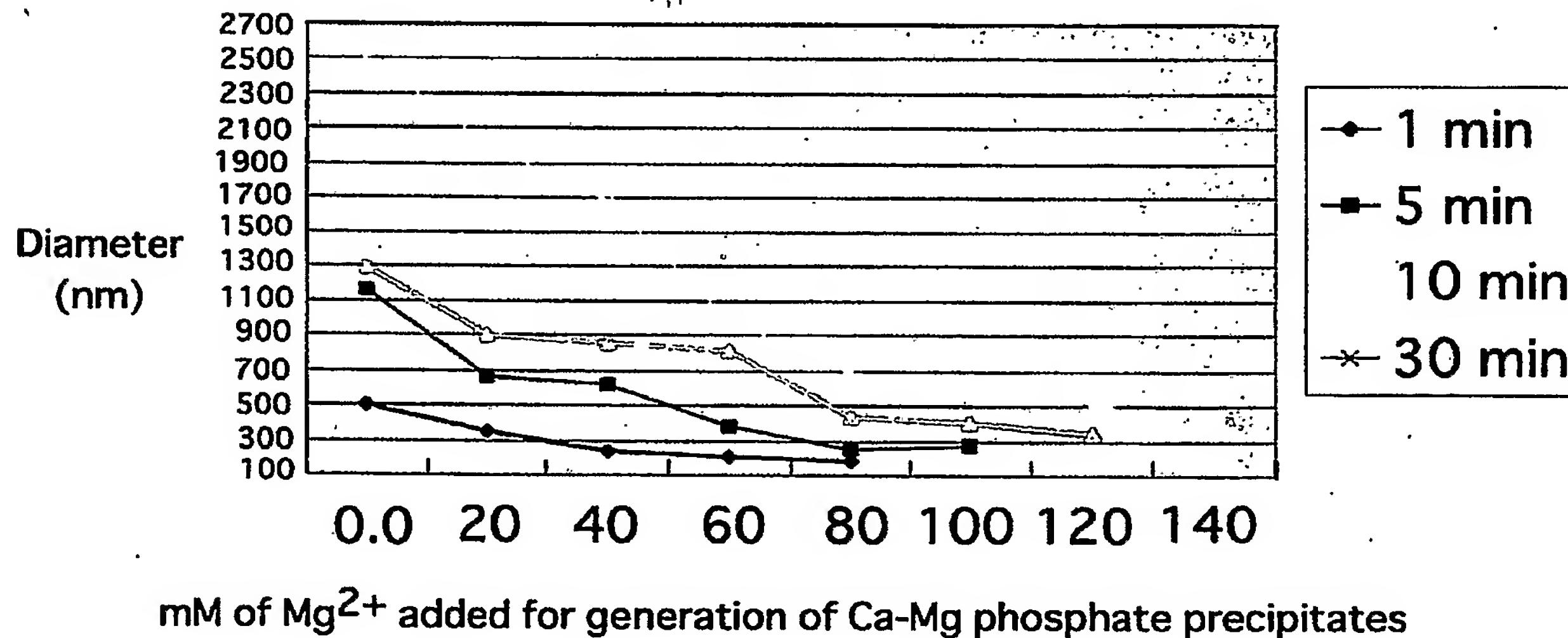
18. Kibalczyc, W., Christoffersen, J., Christoffersen, M. R., Zielenkiewicz, A. and Zielenkiewicz, W. (1990) The effect of magnesium-ions on the precipitation of calcium phosphates. *J. Cryst. Growth* 106, 355-366.
19. Salimi, M. H., Heughebaert, J. C. and Nancollas, G. H. (1985) Crystal growth of calcium phosphates in the presence of magnesium ions. *Langmuir* 1, 119-122.
20. Blumenthal, N. C. (1989) Mechanisms of inhibition of calcification. *Clin Orthop* 247, 279-289.

# Akaike-fig1

A

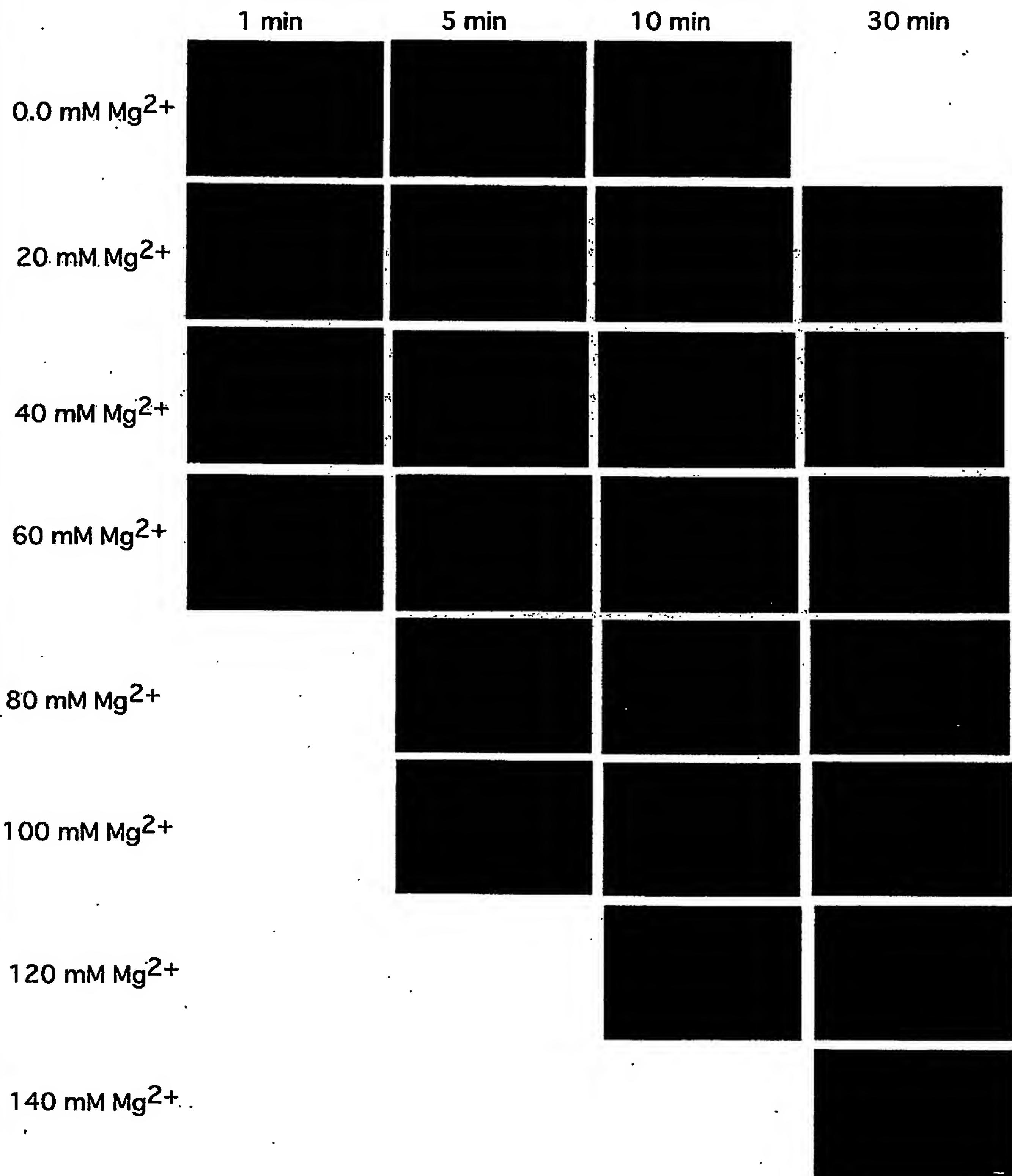


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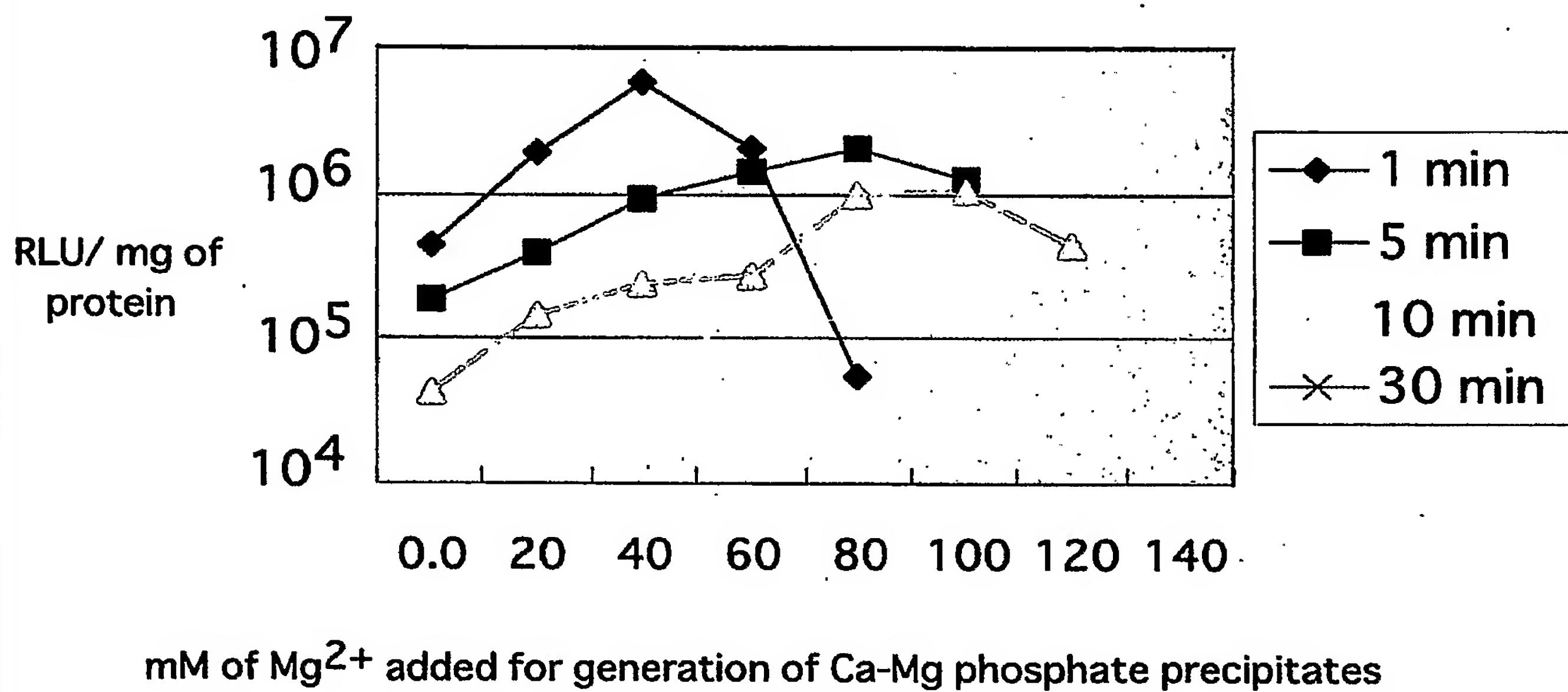
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